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A Gas Liquid Chromatographic Method for the Determination of Phenylalanine in Serum

E. Jellum, V. A. Close, W. Patton, W. Pereira and B. Halpern

Department of Genetics, Lt. J. P. Kennedy, Jr. Laboratories for Molecular Medicine

Stanford University Medical Center, Stanford, California

Running Title: GLC of Serum Phenylalanine

Mailing address for proof:

Department of Genetics
Stanford University Medical Center
Stanford, California 94305

The value of a low phenylalanine diet in preventing the mental defect in phenylketonuric babies is dependent on a simple, rapid and quantitative determination of blood phenylalanine levels. Present analytical methods which depend on chromatographic procedures are either lengthy or only semiquantitative, whilst assays based on the fluorescence obtained by the reaction of phenylalanine with ninhydrin lack the absolute specificity for phenylalanine. (1-4)

We now describe a rapid and specific method for the determination of phenylalanine in plasma or serum using gas liquid chromatography (glc) via the neopentylidene methyl ester derivative. The procedure was developed for use with small amounts of serum (5-50 µl) and for rapid analysis of many samples while maintaining the accuracy and precision of lengthier procedures.

METHODS

Reagents

L--phenylalanine and DL-norleucine were obtained from Mann Research Labs. (N.Y.). Pivaldehyde (2,2-dimethyl propanal) was supplied by K & K Labs (Cal.). The anion exchange resin AG1-X8 (Bio-Rad Labs. (Cal.)) was converted into the bicarbonate form by treatment with sodium bicarbonate (0.5M) followed by washing with water, methanol and dry ether. The resin was dried in vacuum, and kept in a desiccator before use. Linde molecular sieve (3A, 1/16" pellets) was obtained from Matheson, Coleman and Bell (N.J.).

Equipment

Aerograph HiFy Model 600 D gas chromatograph fitted with a 5' x 1/8" column packed with silicone OV-17 on dimethyldichlorosilane treated chromosorb W.

1mV Recorder (Honeywell Electronic 15)

Clinical Centrifuge (International Equipment Comp.)

PROCEDURE

The serum sample (50 µl) is transferred to the bottom of a centrifuge tube (2 ml) and 5 µl of the internal standard (aqueous solution containing 4 mg norleucine/ml) is added with a micropipette. The solution is then deproteinated with alcohol (200 µl) and after centrifugation the supernatent is transferred to a pear shaped flask (1-2 ml) with a Pasteur pipette. The solution is evaporated to dryness in vacuum and the residue is treated with 0.5 ml of thionyl chloride-methanol reagent (from thionylchloride (1 ml) added dropwise to absolute methanol (10 ml) at -25°C). After heating at 85° (oil bath) for 15 minutes the solution is evaporated in vacuum. Methanol (25 µl) and pivaldehyde (5 µl) are added and the solution is made slightly alkaline (pH 7-8) with dry ion exchange resin in the bicarbonate form (5-10 mg of resin). One pellet of molecular sieve is added to remove traces of water and after 5 min part of the sample (1-2 µl) is injected into the gas chromatograph. The analysis were run with a carrier gas flow of 30 ml/min and the temperature was programmed from 150-270°

at 12°/min. Smaller serum samples (∿ 5 µl) have also been used in this assay. In these cases one requires only 1/10th of the reagent quantities described above, and the centrifugation and derivatisation are most conveniently done in small capillaries. Further sample size reduction is possible since the analysis were done at only 1/400th of the maximum sensitivity of the gas chromatograph and 10^{-9} gm of injected phenylalanine can be detected. This compares favorably with the 10^{-8} gm detection limit quoted for the fluorimetric method (3). The amount of phenylalanine present in the serum expressed in mg percent (mg %) is calculated from

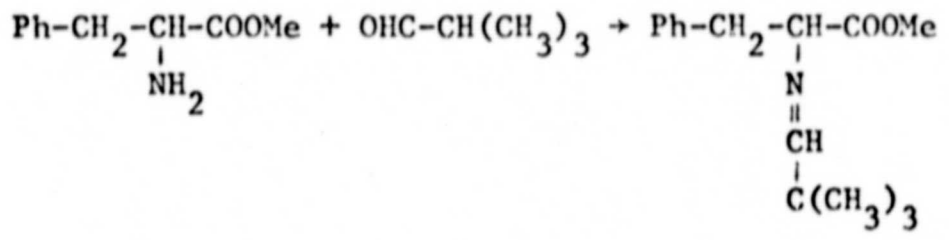
$$\frac{40 \text{ X phenylalanine peak area}}{0.85 \text{ X norleucine peak area}}$$

The factor 0.85 accounts for the different responses of the neopentylidine derivatives of phenylalanine and norleucine methyl esters in the flame ionization detector.

RESULTS

Formation and Chemical Identity of Derivatives.

The derivatisation of phenylalanine for gas liquid chromatography involves the esterification of the amino acid at 85° for 15 min. The ester [I] is then condensed with pivaldehyde [II] to yield the neopentylidene derivative [III]. The formation of [III] is complete after 5 minutes at room temperature at a neutral or slightly alkaline pH.



(I) (II) (III)

To avoid hydrolysis of the hygroscopic ester hydrochloride it is important to carry out the pivaldehyde condensation immediately after the esterification step. The fully derivatised compound [III] is quite stable in anhydrous methanol and can be kept for several days (Table 1).

The relative response of the phenylalanine derivative to the internal standard (norleucine) in the flame ionization detector was established to be 0.85 on a weight basis. This value was obtained by the gas chromatography of known amounts of the distilled fully characterised neopentylidene derivatives (Table 2).

Specificity, Reproducibility and Accuracy

The gas chromatograms obtained from the serum of a normal individual (A) and from a phenylketonuric patient (B) are illustrated in Fig 1. The identification of the glc peaks as amino acid and fatty acid derivatives are based on low resolution mass spectrometry (Finnigan 1015) and the retention behaviour of distilled reference compounds. Further evidence for the correct assignment of the major glc peaks present, was obtained by high resolution mass spectrometry (MS 9) which yielded the molecular formula of

$C_{17}H_{32}O_2$ for methyl palmitate, $C_{19}H_{38}O_2$ for methyl stearate and $C_{19}H_{36}O_2$ for methyl oleate. The homogeneity of the phenylalanine peak in the chromatograms was established with a continuously scanning mass spectrometer, thus demonstrating the absolute specificity of the method for phenylalanine. Fig. 3 demonstrates the linear relationship between phenylalanine concentration and peak area between 0 and 40 mg % of phenylalanine added to normal serum in this range. The serum used in this experiment contained 1.9 mg % of phenylalanine and the curve therefore does not pass through the origin. Table 1 lists the phenylalanine values of 8 separate determinations on a phenylketonuric serum obtained with our method. The greatest source of error is undoubtedly due to the inaccurate measurement of peak areas by the half height method. The quantitative recovery of phenylalanine from a serum sample estimated to contain 29.4 ± 0.6 mg % obtained by our method was further supported by the results of 29.35 ± 0.6 mg % obtained by ion exchange chromatography with a Beckman Model 116 analyzer.

Serum Phenylalanine levels in normal and phenylketonuric individuals.

The serum phenylalanine levels of 5 healthy individuals and 9 phenylketonuric patients on regular diets as determined by the g.l.c. technique are summarized in Table 3. These values in normal and in phenylketonuric people are in good agreement with those in the literature (5, 6, 7) obtained by other methods.

SUMMARY

A rapid, specific and precise gas liquid chromatographic method has been described for the quantitative determination of phenylalanine in serum. The method is based on the conversion of the amino acid to the volatile neopentylidene-phenylalanine methyl ester derivative. It should be useful as a confirmatory test in the diagnosis of suspected phenylketonuria and in the evaluation of the effectiveness of a diet low in phenylalanine.

ACKNOWLEDGMENTS

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TABLE 1

Reproducibility of the GLC-method

Determination No.	Serum Phenylalanine (mg%)
1	25.7
2	26.5
3	27.5
4	27.6
5	26.7
6	27.3
7	27.1
8*	26.8
Mean	26.9
S.D.	0.62

*Chromatographed 3 days after derivatisation.

TABLE 2

Analytical data of neopentylidene amino acid methyl esters

Amino Acid	B.P.	Molecular Formula	Micro Analysis	Mass Spectra Data Characteristic Fragment ($\frac{m}{e}$)
PHENYLALANINE	120°/5 mm	$C_{15}H_{21}O_2N$	Calc: C 72.87, H 8.50, N 5.66 Found: C 72.69, H 8.55, N 5.73	M^+ (247) M^+-CH_2Ph (156) $M^+-COOCH_3$ (188)
NORLEUCINE	70°/5 mm	$C_{12}H_{23}O_2H$	Calc: C 67.71, H 10.79, N 6.57 Found: C 67.37, H 10.79, N 6.55	$M^+-COOCH_3$ (154) $M^+-C(CH_3)_3$ (156)

TABLE 3

Serum phenylalanine values in normals and phenylketonuric patients.

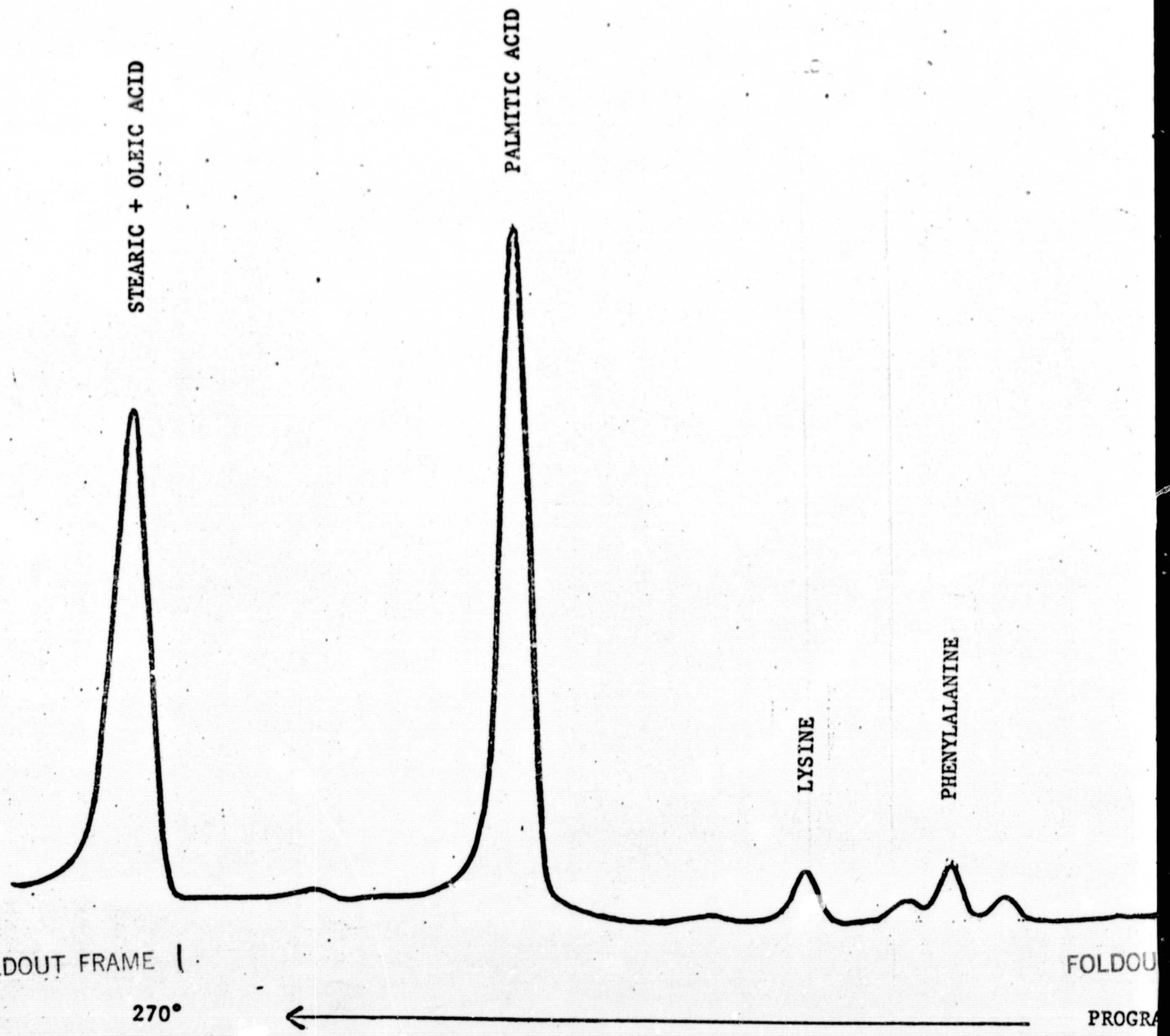
Patient	Serum Phenylalanine (mg%)
1 (Normal)	1.1
2 "	1.3
3 "	1.2
4 "	2.1
5 "	0.6
6 (Phenylketonuric)	26.9
7 "	17.3
8 "	22.1
9 "	33.6
10 "	28.6
11 "	27.5
12 "	14.9
13 "	16.1
14 "	32.9

LEGENDS TO FIGURES

Figure 1. Gas-liquid chromatograms of serum from a normal (A) and from a phenylketonuric (B) patient.

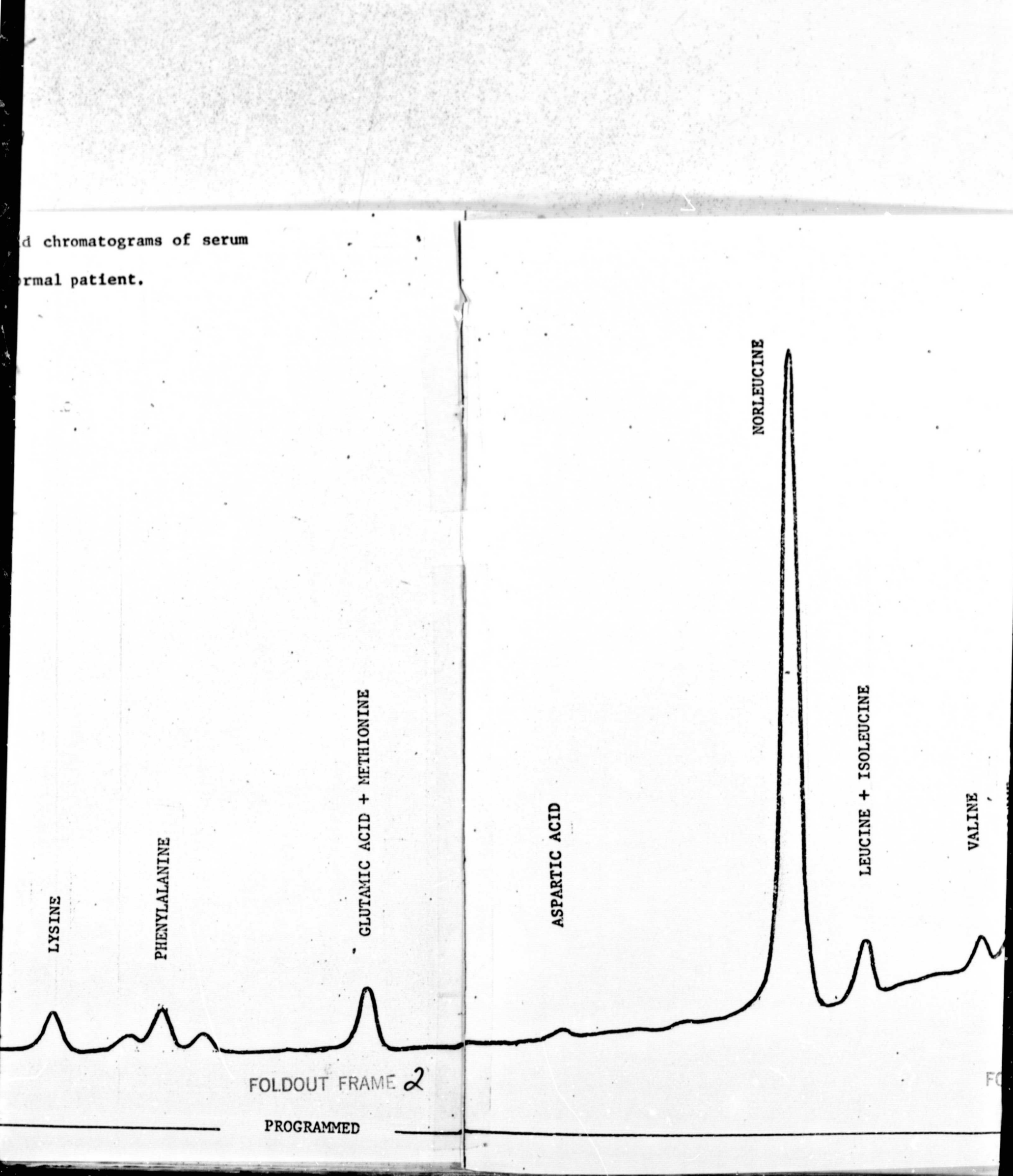
Figure 2. Recovery from serum of added phenylalanine.

Fig. 1A. Gas-liquid chromatograms of serum from a normal patient.



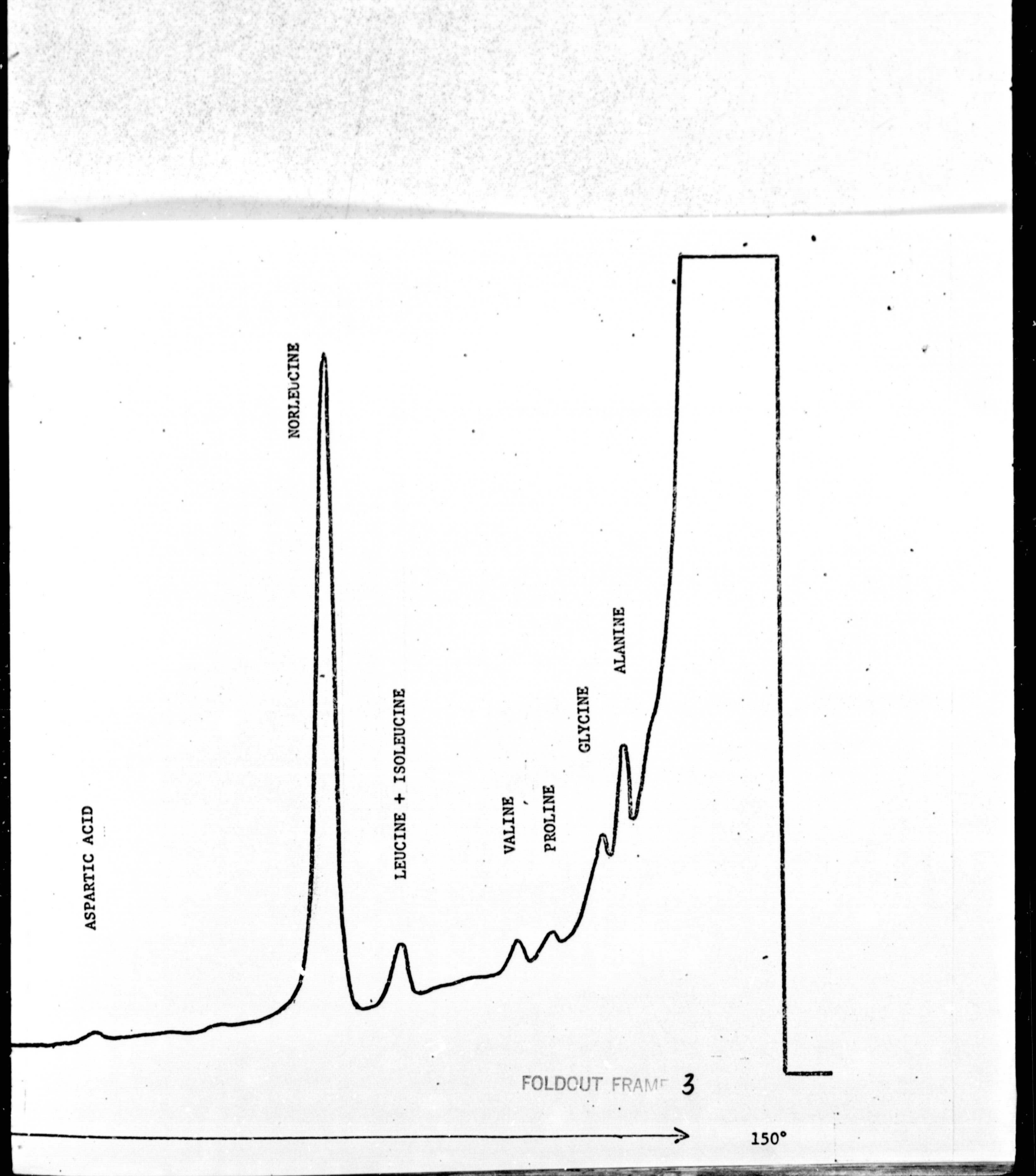
d chromatograms of serum

rmal patient.



FOLDOUT FRAME 2

PROGRAMMED



FOLDOUT FRAME 3

Fig. 1B. Gas-liquid chromatogra

from a phenylketonuri

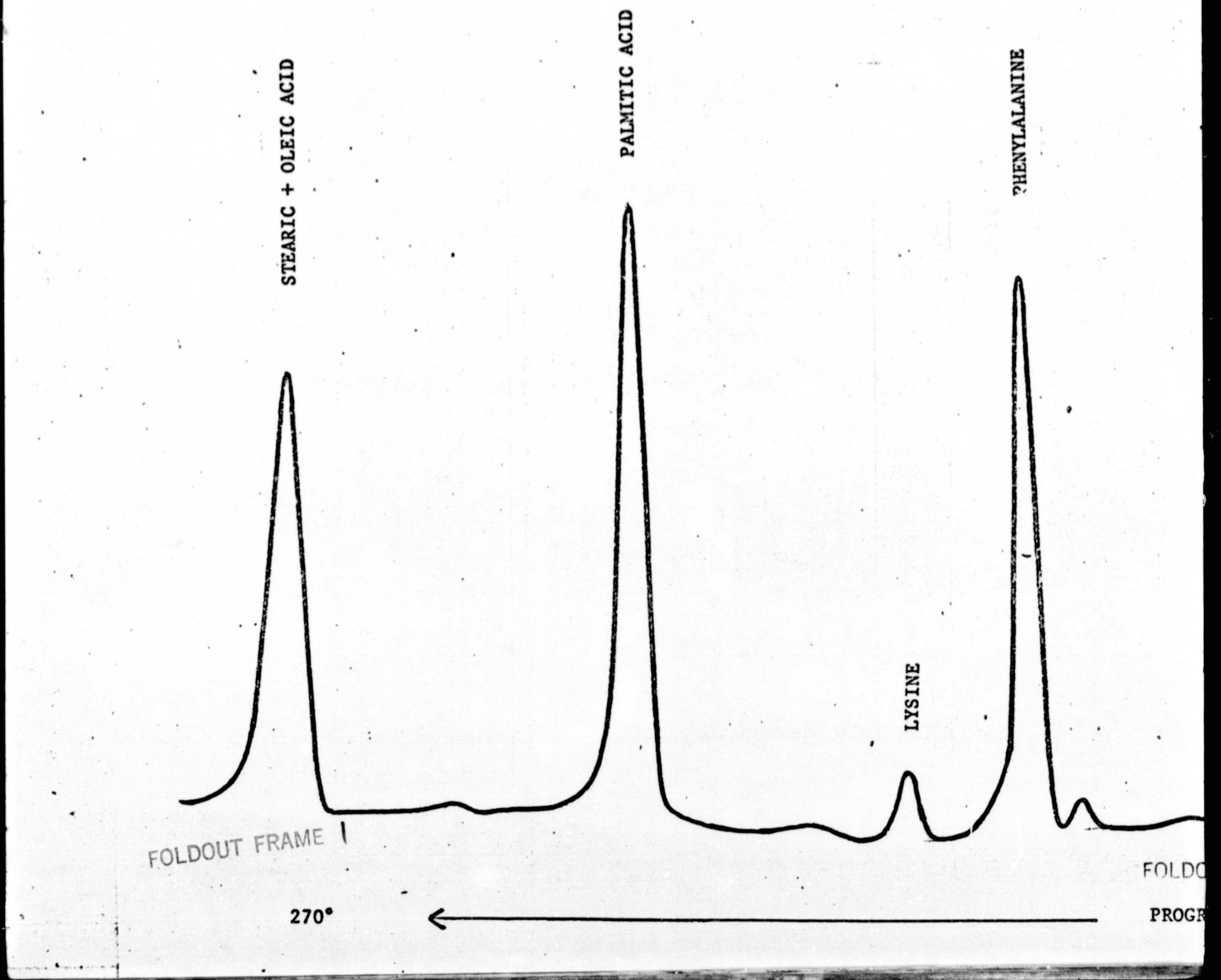
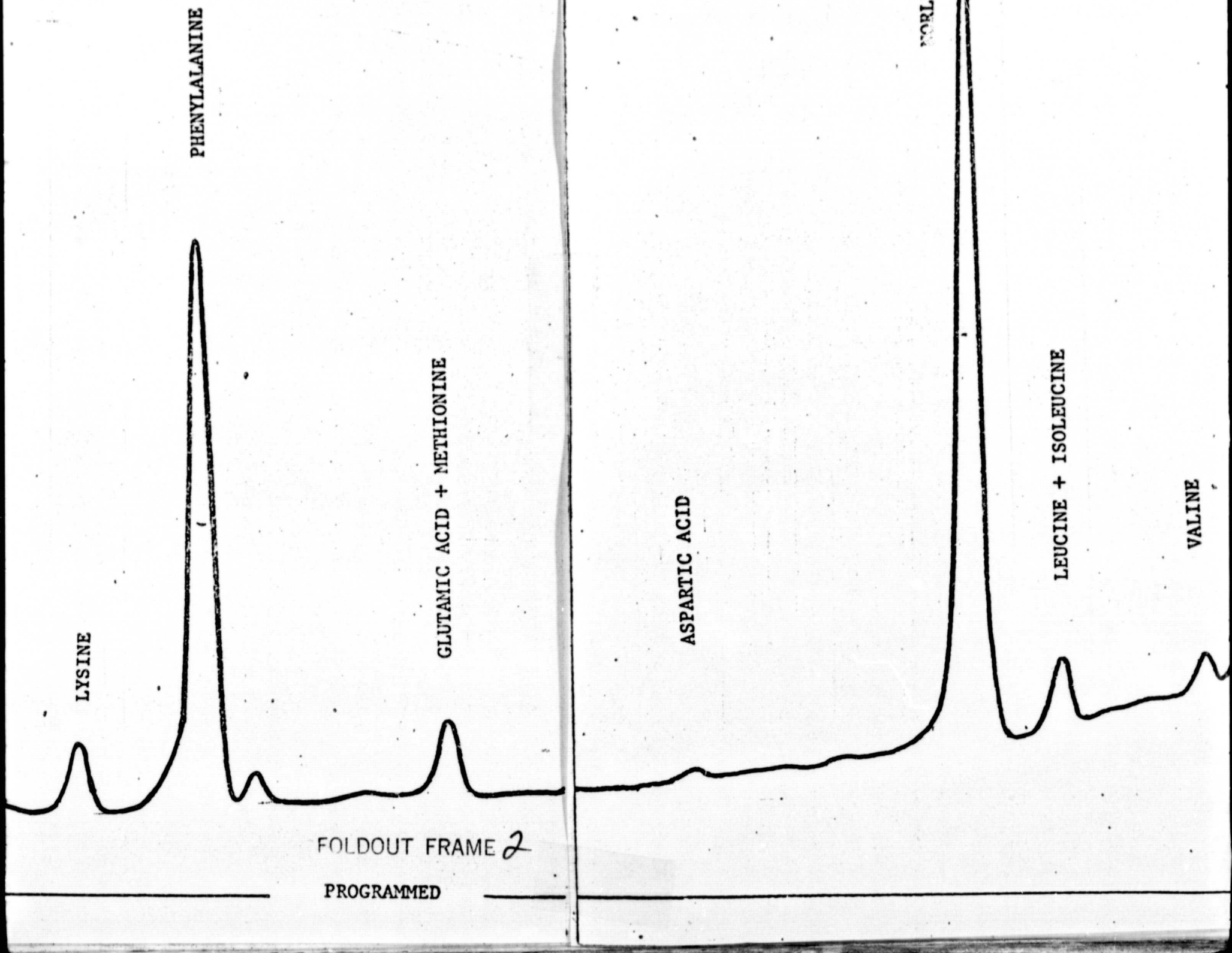
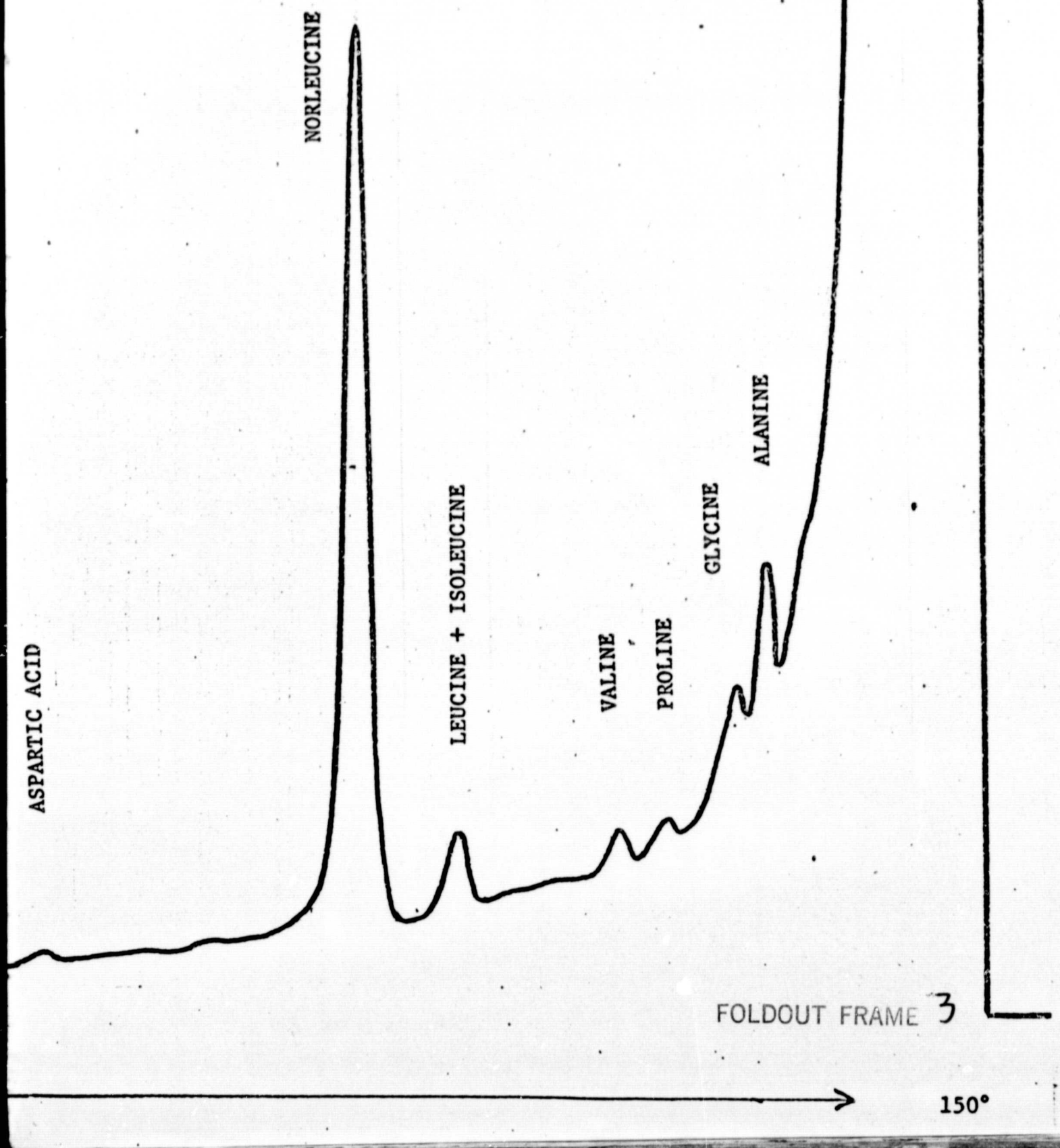


Fig. 1B. Gas-liquid chromatograms of serum from a phenylketonuric patient.




NORLEUCINE
ASPARTIC ACID
LEUCINE + ISOLEUCINE
VALINE
PROLINE
GLYCINE
ALANINE
FOLDOUT FRAME 3
150°

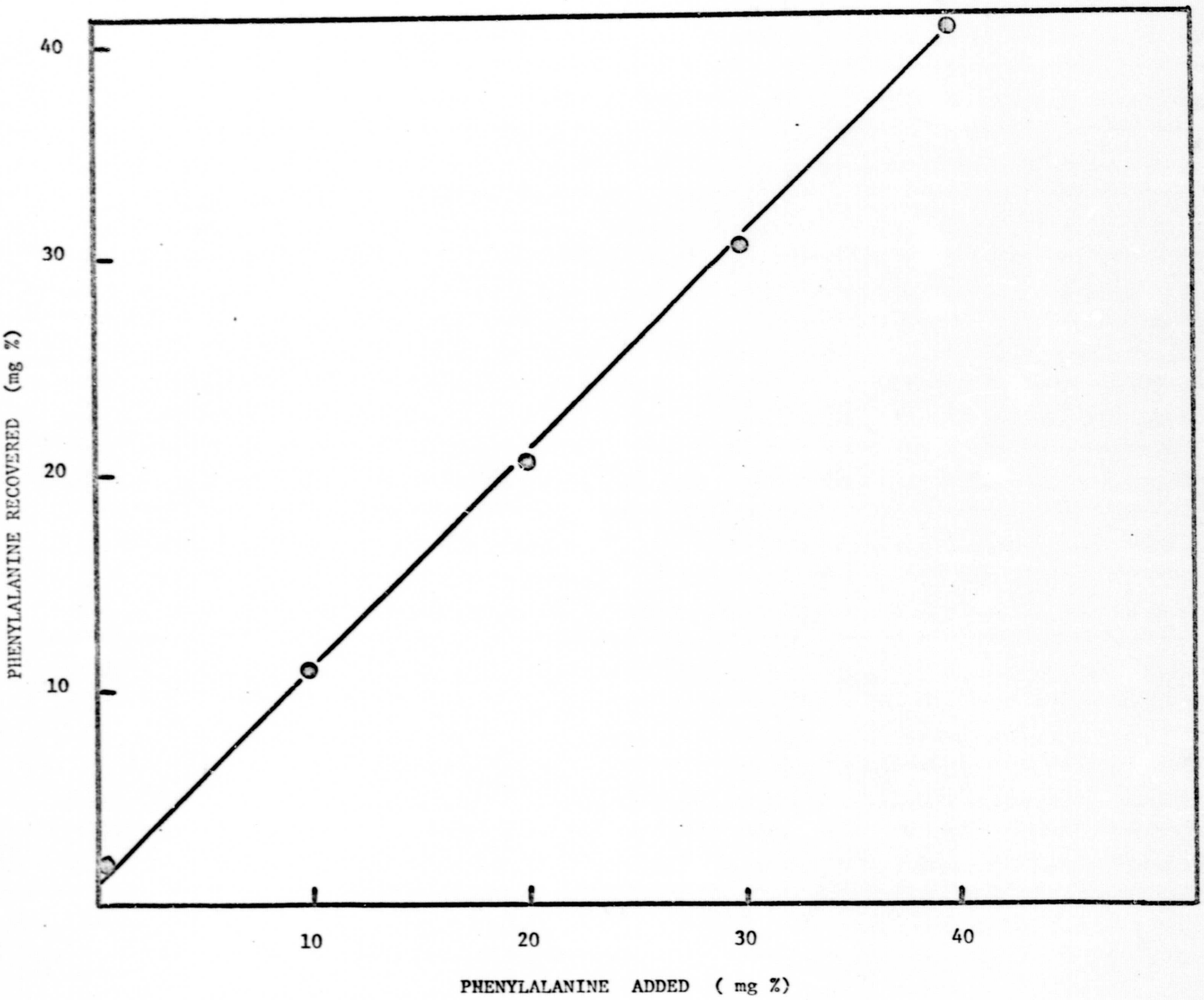

40
30
20
10
PHENYLALANINE RECOVERED (mg %)
10
20
30
40
PHENYLALANINE ADDED (mg %)

Figure 2